Rapid microfluidic dilution for single-molecule spectroscopy of low-affinity biomolecular complexes

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Abstract: To enable the investigation of low-affinity biomolecular complexes with confocal single-molecule spectroscopy, we have developed a microfluidic device that allows a concentrated sample to be diluted by up to five orders of magnitude within milliseconds, at the physical limit dictated by diffusion. We demonstrate the capabilities of the device by studying the dissociation kinetics and structural properties of low-affinity protein complexes using single-molecule two-color and three-color Förster resonance energy transfer (FRET). We show that the versatility of the device makes it suitable for studying complexes with dissociation constants from low nanomolar up to 10 m, thus covering a wide range of biomolecular interactions. The design and precise fabrication of the devices ensure simple yet reliable operation and high reproducibility of the results.

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Rapid microfluidic dilution for single-molecule spectroscopy of low-affinity biomolecular complexes

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To enable the investigation of low-affinity biomolecular complexes with confocal single-molecule spectroscopy, we have developed a microfluidic device that allows a concentrated sample to be diluted by up to five orders of magnitude within milliseconds, at the physical limit dictated by diffusion. We demonstrate the capabilities of the device by studying the dissociation kinetics and structural properties of low-affinity protein complexes using single-molecule two-color and three-color Förster resonance energy transfer (FRET). We show that the versatility of the device makes it suitable for studying complexes with dissociation constants from low nanomolar to ~10 μM, thus covering a wide range of biomolecular interactions. The design and precise fabrication of the devices ensure simple yet reliable operation and high reproducibility of the results.

Single-molecule spectroscopy has developed into a powerful approach for investigating biomolecular structure, dynamics, and interactions, especially in combination with Förster resonance energy transfer (FRET).[1] However, a major challenge for studying the mechanisms of biomolecular interactions by single-molecule spectroscopy, such as FRET between the two binding partners, is that their affinities are often so low that they rapidly dissociate at the ~10 to 100 pM sample concentrations required for single-molecule detection. As a result, single-molecule studies with two fluorescently labeled interaction partners are often not possible at equilibrium. This limitation can be circumvented by forming the complex at high concentrations, then rapidly diluting the sample to single-molecule concentrations and monitor its properties before dissociation.[2] However, there are currently no methods available that combine sufficiently large dilutions and short dead times to enable observation of such complexes before they dissociate. Here we demonstrate a solution to this problem using a microfluidic device that enables the sample to be diluted more than 10,000-fold within milliseconds and allows the properties of the complex and its dissociation kinetics to be monitored by confocal single-molecule spectroscopy.

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A suitable rapid dilution microfluidic device must meet several requirements. First, the dilution needs to be sufficiently rapid that even low-affinity complexes with high dissociation rates can be studied. The minimum time for dilution in laminar flow is fundamentally limited by translational diffusion. Given the diffraction-limited size of the confocal observation volume and the diffusion coefficient of typical biomolecular samples, the minimum dead time for a 10,000 – 100,000-fold dilution, e.g., is in the range of a few milliseconds (Figure S1 in the Supporting Information). Second, since not all applications require the same dilution factor, the device must provide dilutions that can be adjusted over a wide range (~1,000 – 100,000 times). Third, because high sample concentrations are required to form stable biomolecular complexes initially, the device should have low sample consumption. Fourth, the accessible observation times should cover a range from milliseconds to minutes after dilution, so that dissociation kinetics can be monitored and quantified over a wide range of timescales (as required by different dissociation rates). Finally, for a quantitative analysis of the dissociation kinetics, it must be possible to accurately convert the positions throughout the device into times after dilution.[3]

The layout of the microfluidic device is summarized in Figures 1, S2 and S3. Three inlet channels are merged for hydrodynamic focusing of the central sample stream by the two side buffer inlets[4]. In the following broad observation region, the resulting steep concentration profile is expanded and then sectioned into several streams with different sample concentrations by a cascade of microfabricated wedges creating five outlet channels (Figure 1b). To achieve large dilutions, the majority of the sample is diverted to a shunt channel, while the tail of the concentration profile, corresponding to very low sample concentrations, is directed to the observation region (Figure 1c). Figure 1a shows a concentration profile from 3D finite-element calculations of the dilution process. Different positions in the observation region and each of the four long-time observation channels provide access to different dilution factors and thus great flexibility in selecting positions along the streamlines with sample concentrations suited for single-molecule detection. With a total length of about 70 mm, each channel gives access to observation times of up to 3 minutes after dilution. Positions within the channels can be located accurately with the aid of position markers throughout the device.

To attain sufficiently precise structures for accurate and reproducible measurements over a wide range of timescales, we employed precision microfabrication in silicon to generate device molds. Large numbers of devices can then be generated easily

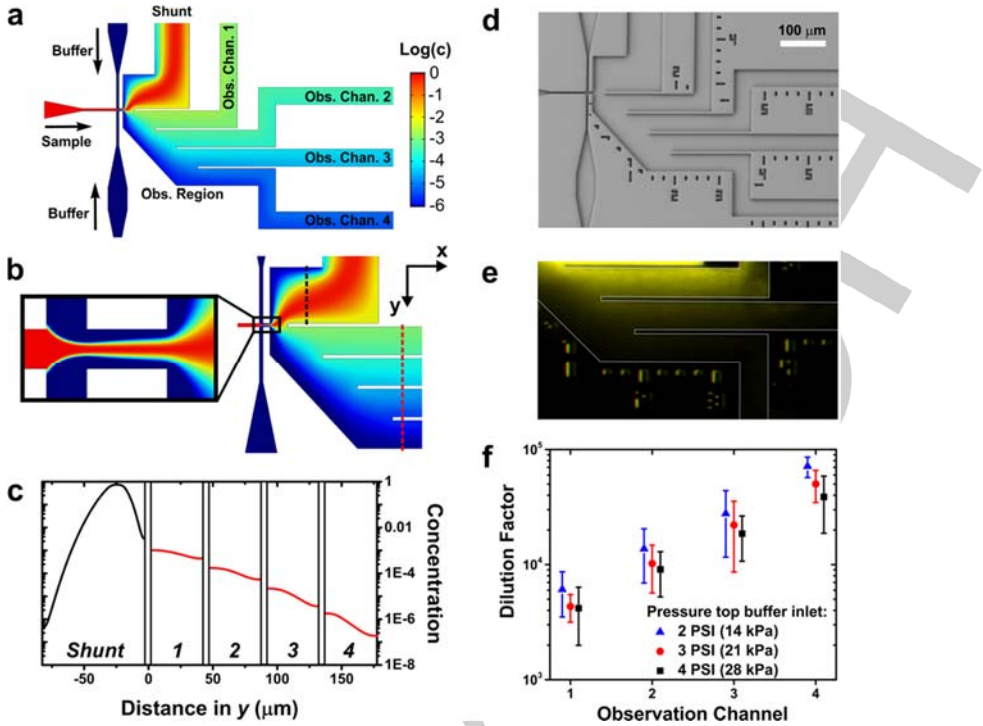

Figure 1: Design and characterization of the microfluidic rapid dilution device. **(a)** Example of a concentration profile based on 3D finite-element calculations of the dilution process. Hydrodynamic focusing of a concentrated sample (red) with buffer (dark blue) results in dilutions covering many orders of magnitude. The concentration is plotted on a logarithmic scale (Log(c)) to visualize the large range of concentrations in a single device. **(b)** Illustration of rapid dilution by concentration sectioning. Hydrodynamic focusing of the center sample stream by two side buffer inlets is employed to generate a sharp concentration gradient across a narrow channel (color scale as in **a**). **(c)** The tail of the concentration distribution is shaved off by the sectioning wedges and directed into the four observation channels. The black and red line represent the cross sections of the concentration distribution in the y direction as indicated by the dashed lines in **b**, where $y = 0$ is at the center of the top sectioning wedge. **(d)** Scanning electron micrograph of the observation region and the cascade of wedges. The markers and numbers are included for locating exact positions in the device. **(e)** Wide-field fluorescence image of the observation channels showing the dilution of 500 μM 20 kDa FITC-Dextran. The outlines of the channels are indicated by white lines. **(f)** Dilution factors for the four observation channels determined by FCS. Error bars are the standard deviation of three measurements in three separate microfluidic devices. By adjusting the pressures applied to the top buffer inlet, the dilution and hence the sample concentrations in the observation channels can be fine-tuned. The data points are offset horizontally for optimal visualization.

with high reproducibility by replica molding using polydimethylsiloxane (PDMS)[5] (Figures 1d and S3) andsubsequent bonding to fused silica cover slides (see Supporting Information for details). Microfabricated filter arrays integrated in each inlet channel (Fig. S2) reduce complications from channel blockage; in combination with computer-controlled electro-pneumatic pressure controllers for driving fluid flow through the channels, stable operation of a single device can typically be maintained for several days.[6] These improvements enable easy-to-use yet robust microfluidic devices for single-molecule experiments with highly reproducible results, as illustrated by the application of previous device designs produced and operated in this way.[7]

The quality of the resulting rapid dilution microfluidic devices is confirmed by the agreement of the finite-element calculations used in the design with the flow velocities measured throughout the microfluidic device with two-focus fluorescence correlation spectroscopy (FCS)[8] (Figures S4 and S5). Further, the sample concentrations in the microfluidic device were quantified using single-focus FCS. As shown in Figure 1f, the design indeed allows the sample to be diluted more than 10,000-fold, thus enabling jumps from micromolar to sub-nanomolar concentrations. The concentration sectioning by the four different observation channels provides direct access to a broad range of different dilutions even at long times without changing the device. By adjusting the pressures applied to the center and side inlets, dilutions of up to 80,000-fold can be achieved.

To benchmark the time resolution of the microfluidic device, we used single-molecule two-color FRET (2c-FRET) to follow the dissociation of a complex of two intrinsically disordered proteins (Figure 2a), the nuclear co-activator binding domain (NCBD) of the CREB binding protein and the activation domain from the p160 transcriptional co-activator (ACTR),[9] which are involved in transcriptional regulation. The complex between donor- (Cy3B) and acceptor- (LD650[10]) labeled ACTR and unlabeled NCBD was formed at protein concentrations of 1 μM, i.e., far above the dissociation constant (K_d = 10 ± 3 nM), and loaded in the center sample inlet (20 μL of sample volume is sufficient to continuously measure for at least 8 hours). In the observation region, positions along the tail of the steep concentration profile were chosen where the sample reaches single-molecule concentrations (~100 pM, i.e., far below the K_d) to monitor the dissociation process (Figure 2b). For accurately extracting the kinetics of dissociation, we used a position-to-time conversion based on time-dependent 3D finite-element calculations[3] (Figure S6 and Supporting Video S1).

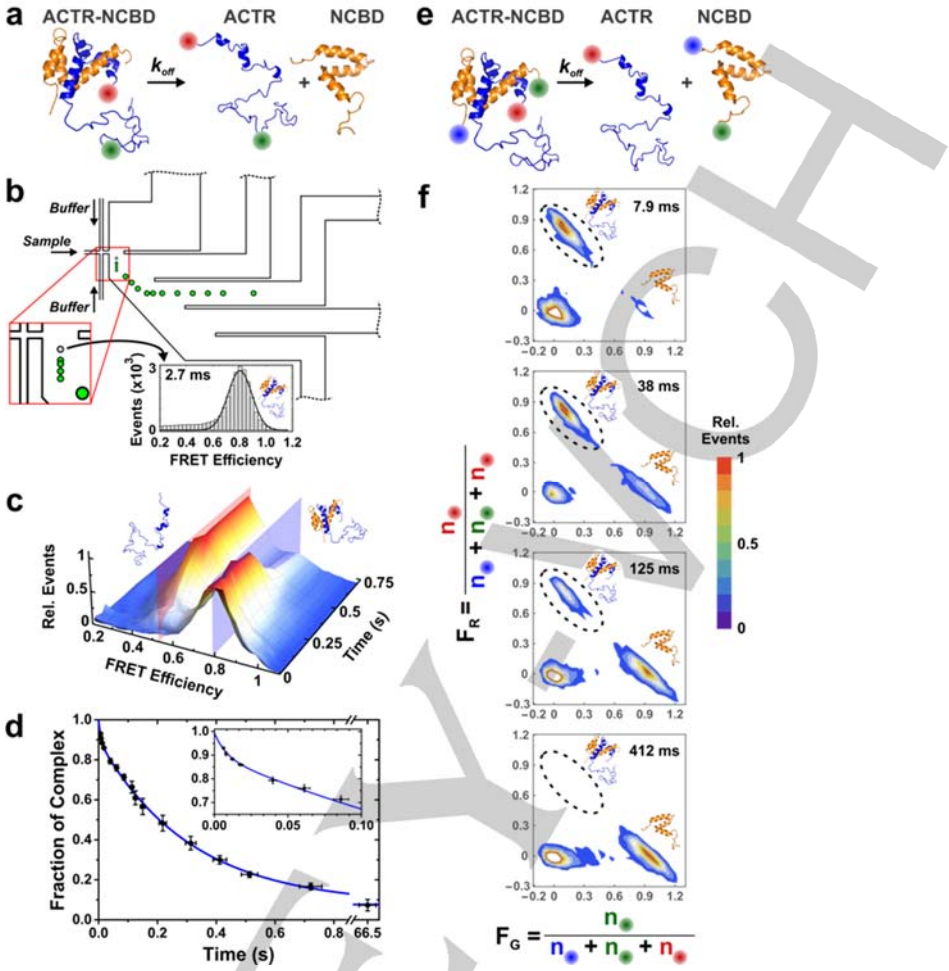

Figure 2: Dissociation kinetics and conformational properties of the ACTR-NCBD complex monitored by 2c- and 3c-FRET **(a, e)** in the rapid dilution device. **(b)** Transfer efficiency histograms were recorded at 16 positions (green circles), each corresponding to a different time after dilution. As early as 2.7 ± 0.3 ms after dilution, a FRET efficiency histogram can be obtained. **(c)** Time series of 2c-FRET efficiency histograms showing that the peak amplitude corresponding to the complex (E = 0.79, blue plane) decreases, while the peak amplitude for unbound ACTR (E = 0.53, red plane) increases with time after dilution. Each histogram is normalized to its maximum value. **(d)** The fraction of complex (black circles) determined from the fits to the transfer efficiency histograms as a function of time after dilution. The uncertainty in the fraction of complex is given by the standard deviation estimated from three independent measurements in separate microfluidic devices. The inset is an expansion of the initial 100 ms, showing the fast decay component. The horizontal error bars were obtained assuming an uncertainty of ± 0.5 µm in detection position and flow velocity variations of ± 5%.[6] The data were fitted with a double-exponential decay (solid blue line). **(f)** Four representative three-color 2D histograms of photon count ratios show that both intra- and intermolecular FRET in the ACTR-NCBD complex (indicated by the dashed ellipse) can be monitored before dissociation. The histograms are normalized to the maximum of the bound and unbound populations.

Figure 2b shows that FRET efficiency histograms can already be recorded 2.7 ± 0.3 ms after dilution. Transfer efficiency histograms recorded at different positions in the observation region and along observation channel 2, corresponding to times after dilution ranging from 6.2 ± 0.6 ms to 66.5 ± 3.3 s (Figures 2b-c and S7), show two relevant peaks: The high transfer-efficiency peak (E = 0.79) corresponds to the protein complex, the peak at E = 0.53 to unbound ACTR. The histograms show that the fraction of complex decreases with increasing time after dilution, as expected. Structural properties, i.e., the mean FRET efficiency, can already be extracted reliably at 2.7 ± 0.3 ms after dilution. However, the low number of photons per burst (resulting from the high flow velocities) and the relatively high sample concentration at these early times limit the accuracy with which absolute populations can be obtained from peak integrals. Both the flow velocity and the sample concentration continue to drop until ~5 ms after dilution, and histograms suitable for accurately determining relative populations can be recorded starting ~6 ms after dilution (Figure S8), close to the dead time dictated by diffusion-limited dilution (Figure S1). To quantify the dissociation kinetics, the fraction of ACTR-NCBD complex was determined for each histogram from the ratio of the area of the high-efficiency peak and the total peak areas. Figure 2d shows the results obtained from three independent measurements in three separate microfluidic devices (see Figure S11 for a comparison of the three datasets). The kinetics deviate from single-exponential behavior, as expected from previous work,[11] and were thus fitted with a double-exponential function. The resulting rate constants, $k_{off,1}$ = 3.2 ± 0.1 s^{-1} and $k_{off,2}$ = 125 ± 88 s^{-1}, are in good agreement with previously reported values.[11a] Note that for the experimental

conditions used here, with a ligand (NCBD) concentration of ~200 pM and a typical association rate constant on the order of 10^8 M^{-1} s^{-1}, the contribution of the association rate to the observed rate is about 0.02 s^{-1}, and is therefore negligible in the observed dissociation kinetics. These results illustrate the fidelity and time resolution of this device. Kinetics are accessible down to the low millisecond range, making the device suitable even for probing protein complexes with low stability and correspondingly large dissociation rates. An example is shown in Figure S12, for a destabilized ACTR-NCBD complex with a dissociation constant K_d of ~8 μM and a corresponding dissociation rate of k_{off} = 540 ± 21 s^{-1}.

This rapid dilution device is thus an enabling technique for studying the structure of low-affinity biomolecular complexes that are inaccessible in equilibrium single-molecule measurements due to their rapid dissociation. Especially for intermolecular FRET experiments, where both binding partners must be fluorescently labeled, rapid dilution of the preformed complex to single-molecule concentrations is required for intermolecular distances to be monitored before dissociation. Here we demonstrate this capability in three-color FRET (3c-FRET) measurements on the ACTR-NCBD complex (K_d = 60 ± 15 nM, Figure 2e). As in the 2c-FRET experiments, preformed complexes between donor- (Alexa Fluor 488) and acceptor- (Alexa Fluor 594) labeled NCBD and acceptor- (Biotium CF680R) labeled ACTR (1 μM) were loaded into the sample inlet. 2D histograms of photon count ratios reporting on intra- (F_G) and intermolecular FRET (F_R) were recorded at positions along the tail of the steep concentration profile where the sample reached single-molecule concentrations, corresponding to times after dilution between 7.9 ± 0.8 ms and 66.5 ± 3.3 s (Figure 2f and S9, for details see Supporting Information). The 2D histograms (Figure 2f) show three peaks. The peak at (F_G = 0.1, F_R = 0.85) corresponds to the complex, the peak at (F_G = 0.9, F_R = 0) to unbound NCBD, and the peak at (F_G = 0, F_R = 0) to molecules lacking an active red or green acceptor dye, respectively.

With increasing time after dilution and corresponding dissociation, the fraction of complex decreases and the fraction of free NCBD increases, as expected. The dissociation kinetics (Figure S10) exhibit a double exponential decay, with rate constants $k_{off,1}$ = 4.3 ± 0.4 s^{-1} and $k_{off,2}$ = 37 ± 10 s^{-1}, which are close to those measured in the 2c-FRET experiments, with small differences presumably resulting from differences in labeling and buffer conditions. With its dissociation on a 100-ms timescale, the complex is clearly inaccessible to observation at single-molecule concentrations by manual dilution. In the rapid dilution device, however, it can be transiently populated, and intra- and intermolecular distance information can be obtained before dissociation occurs.

In summary, we present a novel microfluidic device for single-molecule spectroscopy that is capable of diluting a concentrated sample by almost five orders of magnitude in milliseconds. We show that this device can be used to transiently populate and study the structural properties of low-affinity complexes and to quantify the dynamics of the dissociation process over a wide range of timescales. The design and precision fabrication of the devices ensure simple yet reliable operation on a day-to-day basis and high reproducibility of the results. The versatility of the device in terms of accessible dilutions, long observation times, and short dead time makes it suitable for studying biomolecular complexes with dissociation times down to less than 10 ms. Assuming typical association rate constants of 10^5 - 10^7 M^{-1} s^{-1},[12] this enables the study of complexes with dissociation constants from low nanomolar to >10 μM, covering a wide range of biomolecular interactions.[12b, 13]

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Entry for the Table of Contents

COMMUNICATION

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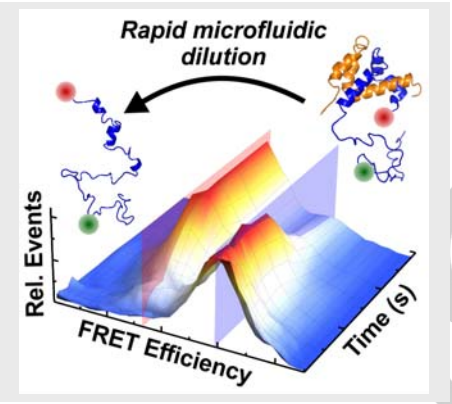

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Page No. – Page No.

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